ON THE HERIDITY OF SOME CHARACTERISTICS IN TWO STRAINS OF ASPERGILLUS FLAVUS-ORYZAE

by

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During a stay in the Treub laboratory, Botanical Gardens, Buitenzorg, I tried to isolate some strains of Aspergillus flavus-Oryzae from the wild. I therefore took some rice which was still in the spike ("padi"); a small quantity of the grains was partly rubbed down and put into an Erlemeyer flask with some distilled water. After a few days, the whole was overgrown by some species of Mucor. I repeated this some times, till in two flasks there appeared, amidst the Mucor, a few tiny flocks of Aspergillus. They could always be found on the bracts, never on the bare starch. From them I oculated on agar and, as soon as growth became visible there, once more on a glucose solution. From that moment the culture was pure (strain R).

In still another way I succeeded in getting Aspergillus flavus-Oryzae, viz. on dead winged termites ("larongs"); when I put half a dozen of them on damp filtering paper in a Petri dish, they were all very soon overgrown by Mucor, save one, which was wholly covered with a yellowish mass of condidia, which looked very much Aspergillus-like. After oculating only once on glucose, this strain was already pure and appeared indeed to be an Aspergillus (strain L).

In order to make sure that I really had to deal with Aspergillus flavus-Oryzae, I identified the strains with

the aid of Wehmer (6), Thom and Church (5) and Boedijn (1). They proved to be:

acc. to Wehmer .....: strain R Aspergillus Oryzae
strain L A. Oryzae or A. flavus
„ „ Thom and Ch. both special forms of A. Oryzae
„ „ Boedijn.....: both a form of A. flavus-Oryzae

(thus they could be directly compared to strain B, which has been dealt with formerly (2) and which very probably is identical with No. 3, described by Boedijn). The "Centraal Bureau voor Schimmelcultures" Baarn, very kindly verified this determination. It confirmed that both strains belong to the group flavus-Oryzae; they both have pitted vesicles and conidia; conidia of R 3—4 μ, of L 5—6 μ; L has got somewhat longer stalks, till 2 m.M., no double sterigmata, as occur in R. Sterigmata in L are grouped more loosely. Most of these morphological differences had been stated by me, nearly a year ago at Buitenzorg, so that they could not have been caused by growing them in the laboratory.

Also the colour of the conidia tallied very well with the statements of the authors mentioned, only in R they were too green to answer satisfactorily to what Thom and Church assume. But I believe that Thom and Church make a mistake, when they state that the colours of the conidia vary from yellow to greenish yellow to brownish yellow, but are not green. A culture, e.g., which on amylum is yellowish brown, can get grass green conidia on a saccharose solution. As I already stated in my former publication, the influence of nutrient liquids must be taken into consideration in this respect.

One might object that the culture, grown on the termites, may be the same as that, which appeared on the rice, as both were obtained in the same room, viz. the Treub laboratory. I do not think, however, that this is the case. Strain R was got out of an abundant mass of Mucor. Now,

if the conidia of R were blown over on the termites, together with the germs of Mucor, how is it that they grew on only one of them, instead of being more or less spread over the others? Aspergillus, however, had that termite entirely to itself, whence I venture to conclude that the conidia had been there before and germinated, as soon as conditions became favourable (i.c. the humidity in the Petri dish). They thus prevented the Mucor from settling on it. The fact, moreover, that in this case I had not the least difficulty to get the culture pure (it was, indeed a pure culture, which arose from that termite), proves to my mind that it is really a strain, different from R. When we compare the physiological conduct of the two strains, this will become even more evident.

Throughout my experiments, differences could be observed between the two strains to the following effect:

The growth of L was, in the beginning, always much slower than that of R; especially during the first week this was very noticeable. After two weeks L had, as a rule, made up the arrears; so, as the dry weight was always determined after the second week and even later, it will not be seen in the tables. But none the less, L had in the beginning always much more difficulty in assimilising the different nutrient solutions.

The colour of the conidia in R was much greener than in L. It was always easily possible to discern, even at a great distance and with the naked eye, between R and L. In R, moreover, the conidia appear much earlier than in L.

Diastase production of R is several times stronger than that of L. At any rate, this is true for any sort of carbohydrate. And if, by way of exception, L reached an equally strong production, it came at least one, if not two weeks later than in R.

R grew extremely slowly on seignette salt, but L did

not germinate at all, though it was oculated several times on a solution of this salt.

It always took more trouble to get the L-cultures out of the flasks, as they clung more or less to the glass wall, which was but very seldom and to a smaller degree the case with R; the culture solution of L filtered, as a rule, much more slowly than that of R. These last mentioned differences may seem rather unimportant, but yet they are striking, because they occurred so constantly throughout the research, which extended over more than 350 cultures.

In one respect the strains were very much alike, as they both produced large quantities of acid. In my former paper I made clear that the formation of acid prevents the hydrolising power of the diastase; I therefore always buffered the nutrient liquids by adding 0.5 % K_2HPO_4, in order to determine the exact quantity of diastase; it appeared that these strains produced too much acid on a 1 % carbohydrate solution, to be neutralised by 0.5 % K_2HPO_4; pH amounted to 4.5 a 5; diastatic action was thus strongly kept down. Therefore I never used carbonic compounds in solutions of more than 0.5 %, at Buitenzorg as well as in Holland.

Only some of the tables, bearing on my investigations, will be given here; in the main, the phenomena are very much like those, published formerly; I only want to emphasize the constancy of the differences between the two strains. As I always grew the fungus on a 0.5 % solution and pH remained about 7.—, I did not think it necessary to mention these data every time in the tables.

Diastase production was determined in the well-known way by means of a diluted solution of soluble potato starch, also by one of rice starch. Dr. B. C. C. J a n s e, director of the chemical department of the Medical Laboratory, Weltevreden, provided me with these substances, which had been prepared for me by his care (by boiling amylum

Table 1.

Diastase production of strains R and L at Buitenzorg, Java.

	R			L		
days	diastase in myc.	diastase in cult. sol.	dry weight in m.Gr.	diastase in myc.	diastase in cult. sol.	dry weight in m.Gr.
			Glucose.			
6	250	25	—	100	5	—
9	—	1250	—	—	25	—
13	—	6000	—	—	750	—
20	9000	3000	—	450	500	—
7	—	1875	113	—	75	79
11	—	22500	83	—	1700	73
25	—	43000	59	—	—	—
			Saccharose.			
7	2500	500	90	2500	50	69
11	15000	3750	117	7000	25	89
25	15000	6000	—	12000	5000	—
			Amylum solubile solani.			
6	—	1850	—	—	3750	—
9	—	1600	—	—	1500	—
13	—	2100	—	—	5000	—
20	—	18000	—	—	28000	—
7	—	14000	88	—	3000	97
11	—	36000	127	—	6000	128
25	—	19000	129	—	—	—
			Amylum solubile oryzae.			
6	—	2500	—	—	700	—
9	—	5000	—	—	500	—
13	—	15000	—	—	9000	—
20	—	16500	—	—	18000	—

solani, resp. amylum oryzae with alcohol and strong sulphuric acid, after which they were filtered and washed). The quantities given, sufficed throughout the research, so the figures from India and Holland can be safely compared.

The data of the diastase production in the tables are those, got by measuring the hydrolytic power with potato starch. But each time it was determined also with rice starch. It appeared invariably that the diastase of Aspergillus flavus-Oryzae hydrolyses this sort of amylum much less easily than potato starch; moreover, the hydrolysis never comes to an end. The colour-changes with jodium from blue to violet and reddish violet are quite normal, though about twice slower than with potato starch; further, the jodium reaction gives very indistinct colours, of an even, dirty violet; colouring into red-orange-yellow was never seen. So the amount of diastase could not be examined in this way and is therefore not recorded in the tables. As this phenomenon appeared in every one of the cultures which were dealt with, it seems worth while stating it here, the more so, as one might easily expect the opposite, rice starch being, so to say, the most natural food stuff of Aspergillus flavus-Oryzae. It would certainly be interesting to go further into this question, by measuring the hydrolysis of the two sorts of starch by the Aspergillus-diastase in a different way, but up to now, I have had no opportunity to do this.

Table 1: These tables need no explanation; only, when we consider the difficulty with which the fungus hydrolyses rice starch, as stated above, attention may be drawn to the fact that, when rice starch is given as food material, the diastase production is by no means less than on potato starch.

Both strains were oculated from the first pure glucose culture on tubes with sterilised rice and with prune agar;

Table 2.

Diastase production of strains R and L in Holland.

number of culture	days	R				L			
		from rice		from prune agar		from rice		from prune agar	
		diast. in cult. sol.	dry weight	diast. in cult. sol.	dry weight	diast. in cult. sol.	dry weight	diast. in cult. sol.	dry weight
		Saccharose.							
	14	1000	169	1250	116	100	110	trace	124
	28	7500	93	1500	102	95	117	40	101
		Amylum solubile solani.							
	9	600	126	1000	73	200	59	180	59
	14	7500	148	18000	89	1000	51	1450	72
	21	6200	106	15000	78	2100	53	2100	73
	35	7500	90	7500	73	750	69	3300	75
		Amylum solubile oryzae.							
	9	1000	107	750	99	560	111	210	64
	14	1250	105	7500	168	7500	141	2500	108
	21	3750	99	9000	135	4300	75	4300	63
	35	5000	82	15000	98	5000	93	4000	82
		Glucose.							
1	15	6000	107	7500	126	75	130	600	156
	22	11000	124	15000	133	150	103	1150	113
2	17	3000	93	13500	87	1750	116	1750	116
	28	15000	109	7600	127	250	119	300	112
3	14	6000	156	11200	144	350	168	1250	130
17	14	13500	123	14000	149	75	110	600	128
18	14	11000	139	15000	100	150	111	600	121
19	17	16000	110	15000	135	125	91	200	113

these were taken to Holland. When they arrived there some two months later, they showed a strong growth on rice, a very scanty one on the prune agar. From that moment, I always oculated parallel series, from the initial rice-, as well as from the prune agar cultures.

On glucose I grew consecutively 19 parallel series; in the next table only the results of the 3 first and the 3 last will be seen, those between showing nothing remarkable.

Table 2: From these tables it appears that the change of climate had no important influence on the physiological conduct of the strains. The diastase production seems indeed to have somewhat gone down (though, esp. in R, it is of the same degree of intensity), but in my opinion, this may be safely ascribed to the lower temperature, at which hydrolysis had to take place (about 20° in Holland, 28° à 30° in India). But the salient point is, that the above described differences between the two strains remain constant throughout the experiments.

In the tables it will be also seen that, contrary to Aspergillus niger, A. flavus-Oryzae is but very little influenced by the after-effect of former nutrient liquids. I stated this already in my other paper. This, however, was only shown as far as carbohydrates (and prune agar) are concerned. The question arose, whether it would be different on albuminous substances, in other words, whether the adaptation of strain L to animal food (the termites) was the cause of its weak diastase production. I therefore grew both strains 15 times consecutively on pepton "Roche" and on an albumine prepared out of blood. After each culture, they were oculated on glucose, so that it can be seen whether, after having been grown 1, 2,.... 15 times on pepton or albumine, the diastase production on glucose is thereby diminished in L or R.

Tables 3 and 4: The data between () bear upon the glucose cultures.

Table 3.

Diastase production of strains R and L on pepton; the numbers between ( ) bear upon the cultures on glucose.

		R		L	
number of culture	days	diastase in culture sol.	dry weight in m.Gr.	diastase in culture sol.	dry weight in m.Gr.
1	14 (17) (28)	375 (3750) (13500)	107 (120) (91)	140 (1500) (125)	91 (89) (78)
2	15 (13) 24	300 (5000) 180	59 (131) 121	190 (400) 150	50 (154) 62
3	17 (13)	200 (15000)	81 (122)	160 (500)	77 (171)
4	13 (14) 23	100 (15000) 100	129 (137) 91	125 (180) 300	130 (166) 103
5	17 (14)	500 (7500)	103 (133)	500 (100)	94 (151)
6	14 (14)	500 (5000)	127 (119)	450 (50)	81 (125)
7	14 (14)	100 (16000)	132 (103)	220 (450)	64 (113)
8	14 (18)	150 (7500)	110 (174)	180 (750)	118 (124)
9	18 (14)	500 (6000)	81 (124)	600 (500)	64 (124)
10	23 (19)	140 (1250)	180 (126)	500 (100)	68 (—)
11	19 (14)	600 (7500)	113 (104)	120 (180)	122 (140)
12	14 (14)	1500 (14000)	156 (130)	110 (150)	113 (127)
13	24 (14)	3000 (8000)	68 (—)	750 (500)	61 (115)
14	21 (14)	1000 (11000)	71 (121)	1000 (200)	71 (—)
15	17 (14)	1000 (12500)	59 (—)	1000 (140)	81 (118)

Table 4.

Diastase production of strains R and L on albumine; the numbers between ( ) bear upon the cultures on glucose.

		R			L		
number of culture	days	diastase in myc.	diastase in cult. sol.	dry weight	diastase in myc.	diastase in cult. sol.	dry weight
1	14 (17)	150	150 (11500)	(126)	125	90 (600)	(124)
2	15 (13) 24	— —	30 (11200) 30	(129)	— —	20 (2500) 60	(126)
3	17 (13)	85	35 (6000)	(161)	100	50 (1250)	(120)
4	13 (14) 23	— 60	60 (7500) 55	(117)	— 125	100 (750) 125	(157)
5	17 (14)	60	60 (9000)	(139)	150	100 (450)	(126)
6	14 (14)	45	25 (13600)	(141)	100	50 (50)	(129)
7	14 (14)	75	50 (2500)	(134)	110	60 (375)	(121)
8	14 (18)	150	30 (7500)	(122)	175	55 (75)	(114)
9	18 (14)	20	45 (6500)	(—)	130	80 (65)	(102)
10	13 (19)	120	100 (7000)	(121)	180	150 (100)	(84)
11	19 (14)	150	110 (11000)	(118)	100	20 (115)	(137)
12	14 (14)	200	120 (11200)	(130)	125	80 (20)	(96)
13	24 (14)	40	25 (18000)	(98)	125	60 (600)	(—)
14	21 (14)	200	180 (9000)	(—)	135	50 (250)	(—)
15	17 (14)	25	20 (11000)	(101)	85	10 (110)	(114)

Even after 15 generations on pepton and albumine, the diastase production on glucose was not influenced in the least. All the differences between the strains remained constant, only the diastase production of R was greatly diminished; often that of L is even greater, though not regularly. Growth on pepton was, as a rule, rather strong, on albumine scanty. If L had been better adapted to this sort of food, it ought to have appeared from the numbers of the dry weight, but the contrary is the case.

The after-effect of former culture solutions is, in the case of Aspergillus flavus-Oryzae, of no practical importance; so the fact that the strains R and L originated from resp. rice and termites, may not be considered the principal cause of their morphological and physiological differences. It might be argued that they had possibly grown wild for already a very long time on their natural sources of food, but on the other hand, the numbers of my consecutive cultures may not be neglected.

For the present, the data indicate that we have to deal with heriditary races. In my former paper I did not yet venture to take this for granted; I had to allow for the unknown influence of origin and change of climate, especially of strain B, as that one in particular differed most from the others, especially in its diastase production. Change of climate may be eliminated now, and origin appears to be of little importance. Though I do not think that absolute proof has been furnished (and I doubt if it ever will be), I am yet inclined to accept the heriditary basis of the differences described and pointed out.

But we must bear in mind that the genus Aspergillus and especially the group flavus-Oryzae present great difficulties on this point. I fully agree with Boedijn, when he writes: "While it is not difficult to arrange them under the big groups of this genus, classification of the frequently

numerous by-forms is practically impossible. Even with the new monograph of Thom and Church, these by-forms cannot satisfactorily be defined" and also: "I have relinquished the idea of giving separate names to the races. (though B. admits that: "Even slight differences in the races proved to be constant in culture"). It is doubtful whether such a thing would be any good in the case of Aspergillus with its numberless forms for every large species. Yet it is not to be denied that it might have been justifiable for a few types in the Aspergillus flavus-Oryzae group".

Yet Boedijn did not give names to the strains of flavus-Oryzae, which he isolated, but simply numbered them 1—7 and think he was right in doing so. If we consider that the two strains, isolated by me, could not be identified with one of those of Boedijn, and further, that there is no reason why any strain, isolated from any spot on earth and from any source of food, should not probably prove to be constantly different from the strains already known, it becomes a hopeless enterprise to give them names. For the present, we have to consider the flavus-Oryzae group as a collection of heriditary races, the number of which cannot be estimated at this moment.

As sexual propagation does not occur in Aspergillus flavus-Oryzae, we can only imagine that they have come into existence by mutation, as Schiemann (4) suggests. This word does not quite satisfy me, as, in my opinion, it only expresses that we do not know how those races arose. The word "Dauermodifikation" from Goldschmidt (3) is perhaps better and more cautious, though I am fully aware that it hardly explains facts. Yet it should not be forgotten that in two cases, it was the strains, isolated from termites (though perhaps accidentally), that appeared to have a slight diastase production, when compared with the others.

Summary.

1. Two wild strains of Aspergillus flavus-Oryzae were isolated from rice and dead termites, at Buitenzorg, Java.

2. The strains showed clearly marked morphological and physiological differences, which remained constant throughout the experiment.

3. The way in which diastase was produced, was the same as described formerly.

4. The hydrolysis of rice starch appeared to be much more difficult for the diastase of Aspergillus flavus-Oryzae than that of potato starch.

5. The strain from the termites did not grow better on pepton and albumine than that from rice; all the other differences appeared constant on these substances, only the diastase production of both was equally reduced and irregular.

6. Change of climate and after effect of different nutrient solutions did not have any influence on diastase production and other characteristics.

7. The facts known at the present moment compel us to consider the strains of Aspergillus flavus-Oryzae as heriditary races.

I wish to express my heartfelt thanks to dr. B. C. C. Janse, director of the chemical department of the Medical Laboratory, Weltevreden, for his kindness in providing me with the soluble starches, necessary for my research; to the "Centraal Bureau voor Schimmelcultures", Baarn, which very kindly verified my determination of the strains, and finally to Mr. C. Deelder, Schiedam, who once more gave his valuable help in correcting the translation of this paper.

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